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Suitable Disk Antimicrobial Susceptibility Breakpoints Defining *Salmonella enterica* Serovar Typhi Isolates with Reduced Susceptibility to Fluoroquinolones▽†‖

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Infections with *Salmonella enterica* serovar Typhi isolates that have reduced susceptibility to ofloxacin (MIC ≥ 0.25 μg/ml) or ciprofloxacin (MIC ≥ 0.125 μg/ml) have been associated with a delayed response or clinical failure following treatment with these antimicrobials. These isolates are not detected as resistant using current disk susceptibility breakpoints. We examined 816 isolates of *S.* Typhi from seven Asian countries. Screening for nalidixic acid resistance (MIC ≥ 16 μg/ml) identified isolates with an ofloxacin MIC of ≥0.25 μg/ml with a sensitivity of 97.3% (253/260) and specificity of 99.3% (552/556). For isolates with a ciprofloxacin MIC of ≥0.125 μg/ml, the sensitivity was 92.9% (248/267) and specificity was 98.4% (540/549). A zone of inhibition of ≤28 mm around a 5-μg ofloxacin disc detected strains with an ofloxacin MIC of ≥0.25 μg/ml with a sensitivity of 94.6% (246/260) and specificity of 94.2% (524/556). A zone of inhibition of ≤30 mm detected isolates with a ciprofloxacin MIC of ≥0.125 μg/ml with a sensitivity of 94.0% (251/267) and specificity of 94.2% (517/549). An ofloxacin MIC of ≥0.25 μg/ml and a ciprofloxacin MIC of ≥0.125 μg/ml detected 74.5% (341/460) of isolates with an identified quinolone resistance-inducing mutation and 81.5% (331/406) of the most common mutant (carrying a serine-to-phenylalanine mutation at codon 83 in the *gyrA* gene). Screening for nalidixic acid resistance or ciprofloxacin and ofloxacin disk inhibition zone are suitable for detecting *S.* Typhi isolates with reduced fluoroquinolone susceptibility.

Enteric fever is an infection caused by *Salmonella enterica* serovars Typhi and Paratyphi A. These human restricted pathogens are transmitted by the fecal-oral route, and enteric fever is common in regions with poor standards of hygiene and sanitation. There are 27 million new enteric fever infections each year, of which approximately 200,000 are fatal (16). Antimicrobials are essential for appropriate clinical management of enteric fever, but antimicrobial resistance in *S.* Typhi and *S.* Paratyphi A have become a problem in regions where they are endemic (6, 8). Multiple-drug-resistant (MDR) *S.* Typhi and *S.* Paratyphi A (resistant to chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin) are particularly common in some locations in Asia and have led to large epidemics. An MDR *S.* Typhi strain was responsible for an outbreak in Tajikistan in the late 1990s, causing over 24,000 infections (39).

The occurrence of MDR strains limits the options for antimicrobial therapy of enteric fever. The current WHO guidelines suggest that the fluoroquinolones are the optimal group of antimicrobials for the treatment of uncomplicated typhoid fever in adults (44). The fluoroquinolones, such as ciprofloxacin and ofloxacin, are comparatively inexpensive and well tolerated and in early randomized clinical trials were very effective. However, *S.* Typhi and *S.* Paratyphi A isolates with reduced susceptibility to fluoroquinolones have become common in Asia and are increasingly common in Africa (6, 8, 13, 26, 32, 37). Infections with *S.* Typhi strains with elevated MICs to ciprofloxacin and ofloxacin have been

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associated with the failure of treatment with these antimicrobials and increased disease severity (15, 30, 33, 36, 43).

Investigations of *S*. Typhi with reduced susceptibility to fluoroquinolones has shown the association of elevated MIC with several single-base-pair mutations in the DNA gyrase gene, *gyrA*, and the topoisomerase gene, *parC* (4, 6, 33, 42). Furthermore, extensive genome sequencing and single nucleotide polymorphism (SNP) investigation of *S*. Typhi strains have further shown the dramatic impact of strains with *gyrA* mutations on the population structure of this monophyletic organism (35). Genotyping studies identified at least 15 independent *gyrA* mutations that have occurred within a decade and stimulated clonal expansion in Asia and Africa (6, 35). These data suggest that such strains have evolved rapidly and are maintained by a strong selective pressure.

The laboratory detection and identification of strains with reduced susceptibility to fluoroquinolones are important for the treating clinician, but such strains are categorized as susceptible by the current interpretive guidelines for fluoroquinolone disk susceptibility testing (3, 11, 19). These isolates are invariably resistant to nalidixic acid, and susceptibility testing with a nalidixic acid disk has been suggested as a suitable screening method for reduced fluoroquinolone susceptibility (11, 19). The British Society for Antimicrobial Chemotherapy (BSAC) has recommended that for invasive isolates of *Salmonella*, an MIC for reduced susceptibility to fluoroquinolones should be determined (3).

Here we have examined the relationship between *gyrA* and *parC* mutations, nalidixic acid resistance, ofloxacin and ciprofloxacin disk inhibition zone sizes, and MIC for a large number of *S*. Typhi clinical isolates from multiple locations in Asia over a 16-year period. We suggest disk susceptibility breakpoints for strains with reduced susceptibility to ciprofloxacin and ofloxacin, which may permit the diagnostic laboratory to detect such isolates and aid the clinical management of enteric fever.

MATERIALS AND METHODS

***S*. Typhi strain collection.** The *S*. Typhi strains used in this study were comprised of isolates collected as part of several independent investigations. The majority of the strains (516 strains) were collected from randomized controlled trials conducted between 1992 and 2002 in southern Vietnam. These trials were conducted using a standard protocol, except for the treatment regimens used, described in detail elsewhere (5, 7, 28, 31, 38, 40, 41). One hundred and four *S*. Typhi strains were isolated as part of a randomized controlled trial (gatifloxacin versus chloramphenicol [ISRCTN53258327]) at Patan Hospital, Kathmandu, Nepal, for the treatment of uncomplicated enteric fever between 2006 and 2008. The remaining *S*. Typhi strains (a total of 196) were collected between 2002 and 2003 as part of population-based prospective surveillance studies conducted by multiple teams in Jakarta, Indonesia ($n = 27$), Dhaka, Bangladesh ($n = 40$), Hechi City, Guang Xi, China ($n = 51$), Kolkata, India ($n = 25$), and Karachi, Pakistan ($n = 53$) (6).

A subset of the strains described above ($n = 100$; from Vietnam, Indonesia, China, India, and Pakistan) and a collection of contemporary *S*. Typhi strains from Vietnam and India ($n = 375$) were additionally selected for screening for *gyrA*, *gyrB*, *parC*, and *parE* mutations. These strains are presented in the supplemental material.

Microbiological methods. The isolates were identified by standard biochemical tests and agglutination with *Salmonella*-specific antisera (Murex Diagnostics, Dartford, United Kingdom). Antimicrobial susceptibilities were tested at the time of isolation by the modified Bauer-Kirby disk diffusion method, with zone size interpretation based on CLSI guidelines (9, 11). Antimicrobial disks tested were chloramphenicol (CHL) (30 μg), ampicillin (AMP) (10 μg), trimethoprim-sulfamethoxazole (SXT) (1.25/23.75 μg), ceftriaxone (CRO) (30 μg), ofloxacin (OFX) (5 μg), and nalidixic acid (NAL) (30 μg). Mueller-Hinton agar and antimicrobial discs were purchased from Unipath, Basingstoke, United Kingdom.

Isolates were stored on Protect beads (Prolabs, Oxford, United Kingdom) at −20°C. The isolates were later subcultured, and the disk antimicrobial susceptibility tests were repeated on Mueller-Hinton agar by CLSI methods for NAL (30 μg), ciprofloxacin (CIP) (5 μg), and ofloxacin (OFX) (5 μg). The zone of inhibited growth for each antimicrobial was measured by three separate investigators blind to the result of the measurements of the others. The average zone size recorded by the three readers was calculated. The MICs for the isolates were determined by the standard agar plate dilution method according to CLSI guidelines or by Etest according to the manufacturer's recommendations (AB Biodisk, Sweden) (10).

The antimicrobials evaluated were CIP (0.008 μg/ml to 4 μg/ml), OFX (0.008 μg/ml to 4 μg/ml), and NAL (0.5 μg/ml to 512 μg/ml). Antimicrobial powders for the agar plate dilution MICs were purchased from Sigma, United Kingdom. The MIC end points were read by two independent investigators, each blind to the result determined by the other. Discrepancies were resolved by discussion. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains for these assays. The results were interpreted according to current CLSI guidelines, susceptible being values of ≤8 μg/ml for nalidixic acid, ≤2 μg/ml for ofloxacin, and ≤1 μg/ml for ciprofloxacin. An isolate was defined as MDR if it was resistant to chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin by disk susceptibility testing.

PCR amplification and sequencing of *gyrA*, *gyrB*, *parC*, and *parE* genes in *S*. Typhi. DNA from the strains that were selected for PCR amplification of the *gyrA*, *gyrB*, *parC*, and *parE* genes was extracted using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's recommendations. Briefly, a single colony was inoculated in 1.5 ml of Luria-Bertani broth and incubated overnight at 37°C with shaking at 300 rpm to reach 10^8 CFU/ml. One ml of the bacterial culture was transferred to a microcentrifuge tube and centrifuged in a microcentrifuge at 13,000 rpm for 2 min. The supernatant was removed, and the bacterial pellet was used for DNA extraction. The extracted DNA was stored at −20°C until required.

Oligonucleotide primers for the amplification of the quinolone resistance-determining regions in *gyrA*, *gyrB*, *parC*, and *parE* genes in *S*. Typhi were as follows (6): *gyrA*, GYRA/P1 (5′-TGTCCGAGATGGCCTGAAGC) and GYRA/P2 (5′-TACCGTCATAAGTTATCCACG) (annealing temperature, 55°C); *gyrB*, StygyrB1 (5′-CAAACTGGCGGACTGTCAGG) and StygyrB2 (5′-TTCCGGCATCTGACGATAGA) (annealing temperature, 62°C); *parC*, StmparC1 (5′-CTATGCGATGT CAGAGCTGG) and StmparC2 (5′ TAA CAGCAGCTCGGCGTATT) (annealing temperature; 62°C); and *parE*, StmparE1 (5′-TCTCTTCCGATGAAGTGCTG) and StmparE2 (5′ ATACGG TATAGCGGCGGTAG) (annealing temperature, 62°C).

Predicted PCR amplicon sizes were 347 bp (*gyrA*), 345 bp (*gyrB*), 270 bp (*parC*), and 240 bp (*parE*). PCRs were performed under the following conditions: 30 cycles of 92°C for 45 s, 55°C or 62°C (depending on the primers) for 45 s, and extension at 74°C for 1 min, followed by a final extension step at 74°C for 2 min.

The DNA sequencing reactions were performed using the CEQ DTCS Quick Start kit (Beckman Coulter) and was sequenced using a CEQ 8000 capillary sequencer, and the resulting DNA sequence was analyzed using CEQuence Investigator CEQ2000XL (Beckman Coulter). All sequences were verified, aligned, and manipulated using Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All *gyrA*, *gyrB*, *parC*, and *parE* sequences were compared to other *gyrA*, *gyrB*, *parC*, and *parE* sequences by BLASTn at NCBI. The DNA sequence of the various *S.* Typhi sequences of *gyrA*, *gyrB*, *parC*, and *parE* were downloaded and aligned with the produced sequences.

Data analysis. Zone size interpretive criteria and interpretive discrepancy rates were calculated by the error rate-bounded method of Metzler and DeHaan (27). The MIC breakpoints for reduced susceptibility were ≥0.25 μg/ml for ofloxacin and ≥0.125 μg/ml for ciprofloxacin. The zone size breakpoints were adjusted until the number of false-susceptible disk diffusion test results (very major discrepancies) and false-resistant disk tests (major discrepancies) were held to a minimum. Guidelines for acceptable discrepancy rates were according to the CLSI recommendation (12). Normally distributed data were compared using the Student *t* test, nonnormally distributed data using the Mann-Whitney U test, and proportions by the chi-square test. Statistical analysis was performed using EpiInfo, version 6 (CDC, Atlanta, GA), and SPSS for Windows version 10.1 (SPSS, Inc., Chicago, IL).

RESULTS

Antimicrobial susceptibility testing of *S*. Typhi isolates. We investigated 816 *S*. Typhi isolates collected between 1992 and 2008 from seven Asian countries: Vietnam, Nepal, Indonesia, India, Bangladesh, Pakistan, and China. Only one isolate (the

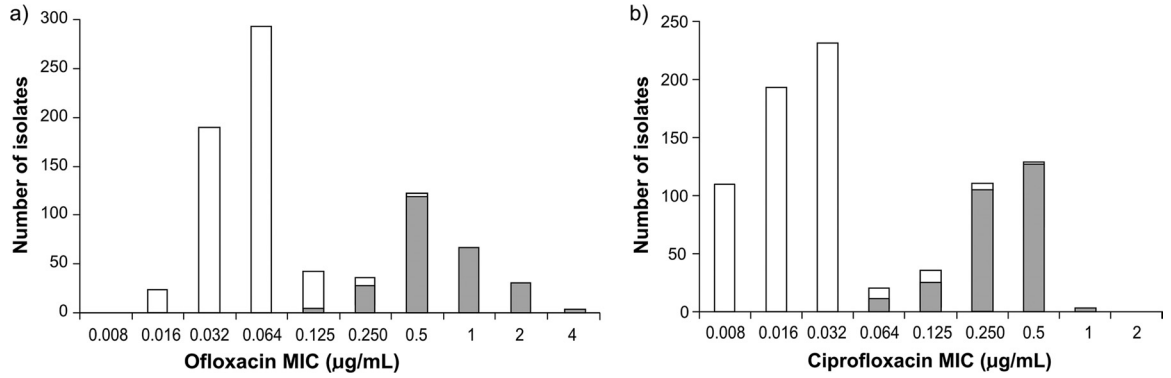

FIG. 1. Fluoroquinolone MIC histograms for 816 *S*. Typhi isolates from Asia. Histograms showing the distribution of MICs to ofloxacin (a) and ciprofloxacin (b) of 816 *S*. Typhi strains, isolated from patients with enteric fever. Each isolate used for analysis was isolated from an individual enteric fever patient. The MICs are plotted on the *x* axis, and the numbers of isolates corresponding with particular MICs are plotted on the *y* axis. The white proportion of the columns indicates the nalidixic acid-susceptible isolates ($n = 563$). The black proportion of the columns indicates the nalidixic acid-resistant isolates ($n = 253$). Both histograms show a bimodal distribution, which is partly differentiated by nalidixic acid resistance.

strain isolated on admission to the health care facility) from each patient was included for microbiological examination and analysis.

Of the 816 *S*. Typhi isolates tested, 466 (57.1%) were MDR (resistant to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole), while 303/816 (37%) were fully susceptible to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole. Two hundred fifty-three of the 816 isolates (31%) were resistant to nalidixic acid (MIC, ≥32 μg/ml), and 4 isolates had an MIC of 16 μg/ml (intermediate) to nalidixic acid but were classified as resistant according to the zone sizes from disk susceptibility testing (≤13 mm). Of the 466 MDR isolates, 145 (31.1%) were additionally resistant to nalidixic acid compared to 80/303 (26.4%) isolates that were fully susceptible to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole ($P = 0.16$).

All 816 *S*. Typhi isolates were classified as susceptible to ciprofloxacin according to MIC testing (MIC ≤ 1 μg/ml), yet 12 gave a discrepant result with disk testing. These strains exhibited an inhibition zone size of ≤20 mm and were, therefore, classified as intermediate by disk testing. Two of the 816 *S*. Typhi strains were graded with intermediate resistance to ofloxacin with an MIC of 4 μg/ml but had inhibition zone sizes of ≥16 mm and were, therefore, classified as susceptible.

The distribution of the MIC levels to ciprofloxacin and ofloxacin for all 816 *S*. Typhi isolates is presented in Fig. 1. The histograms of the levels of MIC to ciprofloxacin and ofloxacin both demonstrate a bimodal distribution. The two distinct groups are partially divided by nalidixic acid susceptibility (Fig. 1, black shading denotes resistance to nalidixic acid). The 563 isolates that were susceptible to nalidixic acid had an MIC_{90} (range) to ciprofloxacin of 0.03 μg/ml (0.008 to 0.5 μg/ml) and of 0.06 μg/ml (0.016 to 0.5 μg/ml) to ofloxacin. The 253 isolates that were resistant to nalidixic acid had an MIC_{90} (range) to ciprofloxacin of 0.5 μg/ml (0.064 to 1 μg/ml) and to ofloxacin of 1.0 μg/ml (0.125 to 4 μg/ml).

Antimicrobial susceptibility test interpretive categories of *S*. Typhi to ciprofloxacin and ofloxacin. The current CLSI intermediate breakpoints are 2 μg/ml and 4 μg/ml, respectively, for ciprofloxacin and ofloxacin. Only 2 of the 816 strains tested had MIC levels greater than or equal to those of the current MIC breakpoints (Fig. 1). The MICs for nalidixic acid were compared with those of ofloxacin and ciprofloxacin in scatter plots (Fig. 2). The current interpretive breakpoints are shown in Fig. 2 as dark shading in red for ofloxacin and ciprofloxacin and in gray for nalidixic acid. The suggested interpretive breakpoints for reduced susceptibility are depicted by a broken line with an arrow (Fig. 2). As predicted, there was a linear relationship between the nalidixic acid MIC and the ofloxacin (Fig. 2a) and ciprofloxacin MICs (Fig. 2b).

Screening strains using nalidixic acid resistance (MIC ≥ 16 μg/ml) for the detection of isolates with an MIC of ≥0.25 μg/ml for ofloxacin had a sensitivity of 97.3% (253/260) and a specificity of 99.3% (552/556) (Fig. 2a). The number of very major discrepancies was 7/260 (2.7%), with none more than two dilutions above the breakpoint, and the number of major discrepancies was 4/556 (0.7%), with none more than two dilutions below the breakpoint. Screening for the detection of isolates with a ciprofloxacin MIC of ≥0.125 μg/ml, using nalidixic acid resistance (MIC of ≥16 μg/ml), was not as reliable as that for ofloxacin, as it had a sensitivity of 92.9% (248/267) and a specificity of 98.4% (540/549). The number of very major discrepancies was 19/267 (7.1%), with 1/267 (0.4%) more than two dilutions above the breakpoint, and the number of major discrepancies was 9/549 (1.6%), with none more than two dilutions below the breakpoint.

We explored the relationship between the diameter of the zone of inhibition and the MICs for ciprofloxacin and ofloxacin, using 5-μg disks (Fig. 3). A zone of inhibition of ≤28 mm around a 5-μg ofloxacin disk correlated with an MIC of ≥0.25 μg/ml, with the least number of discrepancies (Fig. 3a). The number of very major discrepancies was 14/260 (5.4%), with none more than two dilutions above the breakpoint, and the number of major discrepancies was 32/556 (5.7%), with 14/556 (2.5%) more than two dilutions below the breakpoint. A zone of inhibition of ≤28 mm around a 5-μg ofloxacin disc detected strains with an ofloxacin MIC of ≥0.25 μg/ml, with a sensitivity of 94.6% (246/260) and a specificity of 94.2% (524/556). A zone of inhibition of ≤30 mm around a 5-μg ciprofloxacin disk correlated with an MIC of ≥0.125 μg/ml, with the least num-

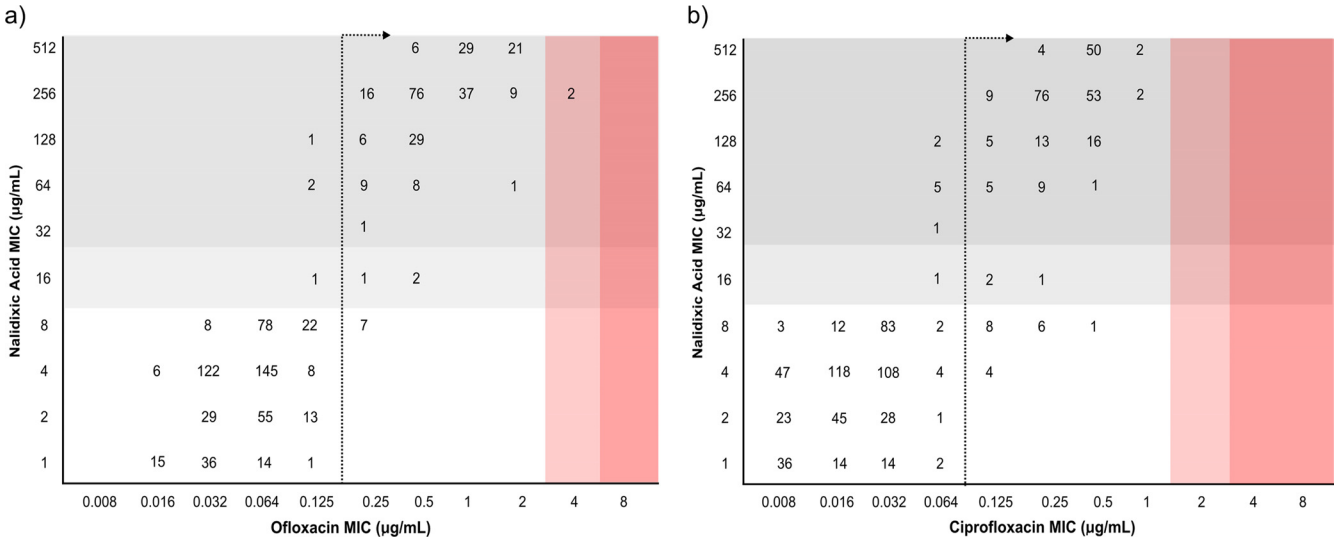

FIG. 2. Scatter plots relating ofloxacin and ciprofloxacin MICs to nalidixic acid MIC for 816 Asian *S*. Typhi isolates. Scatter plots comprised of MIC data from 816 *S*. Typhi isolates from Nepal ($n = 104$), India ($n = 25$), Indonesia ($n = 27$), Bangladesh ($n = 40$), Pakistan ($n = 53$), China ($n = 51$), and Vietnam ($n = 516$). Plots show the relationship between the MIC to nalidixic acid (*y* axis) and the MIC to ofloxacin (a) and ciprofloxacin (b) (*x* axis). The vertical and horizontal shading in each scatter plot indicates the current CLSI recommendations for breakpoints between susceptibility (white), intermediate (light gray, nalidixic acid; light red, ofloxacin and ciprofloxacin), and resistance (dark gray, nalidixic acid; dark red, ofloxacin and ciprofloxacin) (nalidixic acid MIC, ≤8 μg/ml and ≥32 μg/ml; ofloxacin MIC, ≤2 μg/ml and ≥8 μg/ml; and ciprofloxacin MIC, ≤1 μg/ml and ≥4 μg/ml). The red broken line corresponds to the proposed MIC breakpoint identifying strains with reduced susceptibility to fluoroquinolones (ofloxacin MIC of ≥0.25 μg/ml and ciprofloxacin MIC of ≥0.125 μg/ml).

ber of discrepancies (Fig. 3b). The number of very major discrepancies was 16/267 (6.0%), with 4/267 (1.5%) more than two dilutions above the breakpoint, and the number of major discrepancies was 32/549 (5.8%), with 22/549 (4.0%) more than two dilutions below the breakpoint. A zone of growth inhibition of ≤30 mm detected isolates with a ciprofloxacin MIC of ≥0.125 μg/ml, with a sensitivity of 94.0% (251/267) and a specificity of 94.2% (517/549).

Reduced susceptibility to fluoroquinolones and *gyrA*, *gyrB*, *parC*, and *parE* mutations. To further define the *S*. Typhi population with reduced susceptibility to fluoroquinolones, we produced PCR amplicons and then sequenced the quinolone resistance-determining region in the *gyrA*, *gyrB*, *parC*, and *parE* genes from a collection of 475 *S*. Typhi strains from Vietnam, China, India, Indonesia, and Pakistan. One hundred of these strains were described in the previous section, and 375 were more recent strains from Vietnam and India. The MIC range of these strains was 1 to 512 μg/ml to nalidixic acid, 0.008 to 6 μg/ml to ciprofloxacin, and 0.03 to 12 μg/ml to ofloxacin. These strains and the corresponding data from these strains are described in the supplemental material.

Fifteen of the 475 *S*. Typhi strains examined by PCR and sequencing of *gyrA*, *gyrB*, *parC*, and *parE* had no mutations in the quinolone resistance-determining regions of any gene. No strains had a mutation in the quinolone resistance-determining region of *gyrB* or *parE*. Four hundred sixty strains had either a single mutation or a combination of double or triple mutations in the *gyrA* and *parC* genes. DNA sequencing identified seven different amino acid substitutions: D87A, aspartic acid to asparagine at codon 87 in the *gyrA* gene; S83Y, serine to tyrosine at codon 83 in the *gyrA* gene; S83F, serine to phenylalanine at codon 83 in the *gyrA* gene; D87G, aspartic acid to glycine at codon 87 in the *gyrA* gene; S83F/D87N, serine to phenylalanine at codon 83 and aspartic acid to asparagine at codon 87 in the *gyrA* gene; S83F/D87G, serine to phenylalanine at codon 83 and aspartic acid to glycine at codon 87 in the *gyrA* gene; and S83F/D87G/S80I, serine to phenylalanine at codon 83 and aspartic acid to glycine at codon 87 in the *gyrA* gene and serine to isoleucine at codon 80 in the *parC* gene. The most commonly identified amino acid replacement was S83F, constituting (88%) 406/460 strains with a mutation, with S83Y the second most common mutant (10%) 46/460.

We compared the MICs to ofloxacin and ciprofloxacin of the 460 strains with the seven different mutation patterns and the 15 strains with no mutation detected (Fig. 4). When grouped into strains with and without a single mutation in the *gyrA* gene, the single mutation group had significantly higher MICs to ofloxacin (Fig. 4a) and ciprofloxacin (Fig. 4b) than those without a mutation. The most common amino acid substitution, S83F, had mean MICs of 0.75 μg/ml and 0.33 μg/ml to ofloxacin and ciprofloxacin, respectively. Figure 4 also shows the current CLSI breakpoints and the suggested ofloxacin breakpoint of 0.25 μg/ml and ciprofloxacin breakpoint of 0.125 μg/ml. An MIC of 0.25 μg/ml to ofloxacin and an MIC of 0.125 μg/ml to ciprofloxacin detected 74.5% (341/460) of the *S*. Typhi strains with an identified fluoroquinolone resistance mutation and 81.5% (331/406) of the most common *S*. Typhi mutant (S83F) with reduced susceptibility to fluoroquinolones.

DISCUSSION

The increasing recognition that *S*. Typhi isolates with reduced susceptibility to ofloxacin and ciprofloxacin may lead to treatment failure has led to calls for a revision of their break-

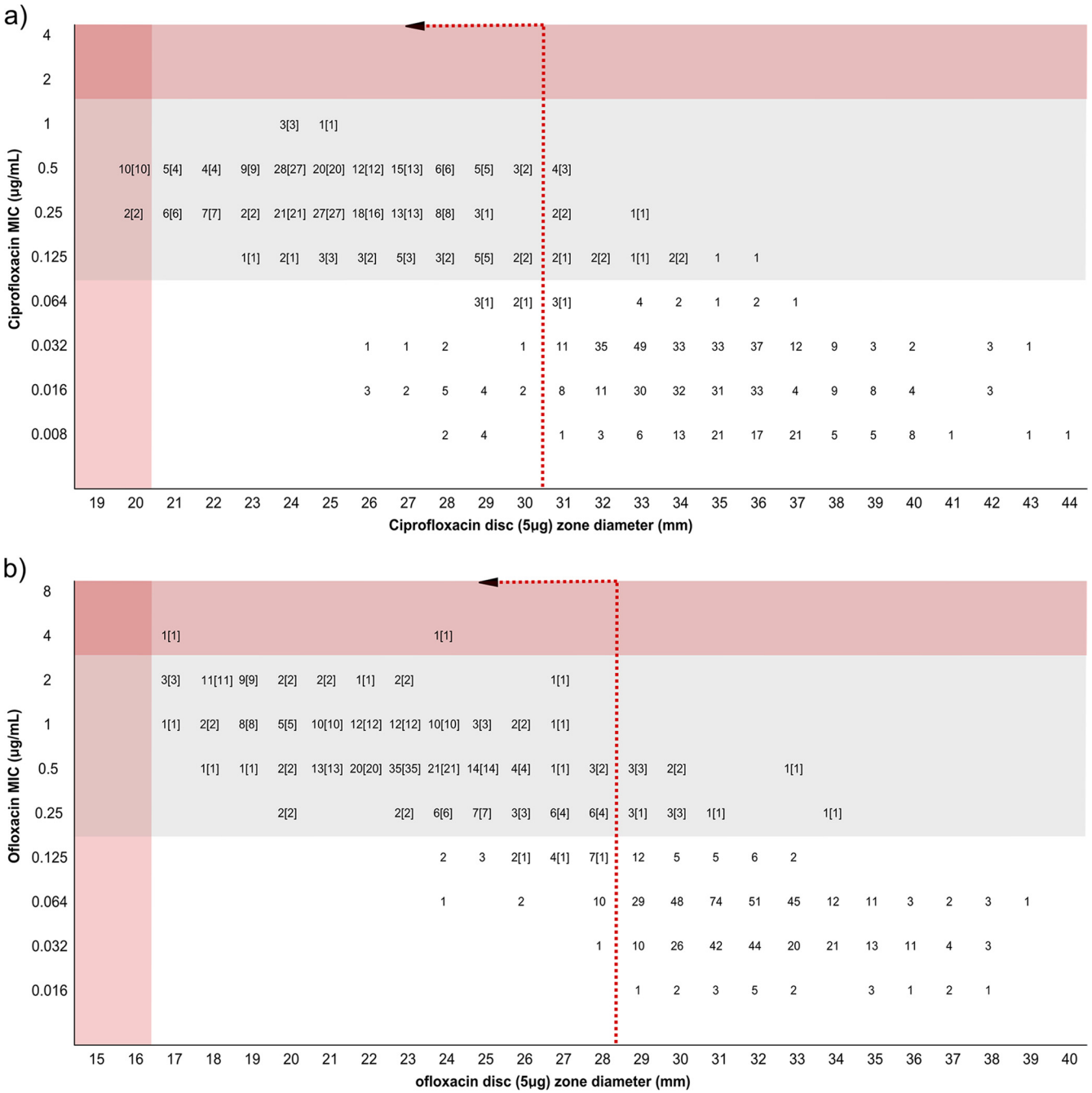

FIG. 3. Scatter plots relating ofloxacin and ciprofloxacin MIC to inhibition zone diameter for 816 Asian *S*. Typhi isolates. Scatter plots for 816 *S*. Typhi isolates comparing the inhibition zone diameters using a 5-μg ciprofloxacin disc (a) and a 5-μg ofloxacin disc (b) (*x* axis) and the corresponding MIC of ciprofloxacin (a) and ofloxacin (b) (*y* axis). The numbers in brackets relate to the 253 nalidixic acid-resistant isolates. The vertical red shading in each scatter plot is the current CLSI disc zone breakpoint for resistance (ofloxacin inhibition zone diameter, ≤16 mm; ciprofloxacin inhibition zone diameter, ≤21 mm). The horizontal red shading distinguishes strains with an MIC of ≥2 μg/ml for ofloxacin or an MIC of ≥1 μg/ml for ciprofloxacin. The gray shading is the proposed breakpoint for *S*. Typhi isolates with reduced susceptibility (ofloxacin MIC, ≥0.25 μg/ml; ciprofloxacin MIC, ≥0.125 μg/ml). The red broken line corresponds with the proposed breakpoints for strains with reduced susceptibility (ofloxacin inhibition zone diameter, ≤28 mm; ciprofloxacin inhibition zone diameter, ≤30 mm).

points. Breakpoints of ≥0.25 μg/ml for ofloxacin and levofloxacin and ≥0.125 μg/ml for ciprofloxacin and gatifloxacin have been suggested (1, 2, 14, 32). Nalidixic acid resistance and disk susceptibility testing have both been proposed as laboratory screening methods to detect such isolates. We have explored the performance of these methods with a large number of strains that are representative of *S*. Typhi isolates circulating in countries in Asia where it is endemic.

Nalidixic acid resistance had a sensitivity of 96.2% and 91.8% and a specificity of 99.5% and 98.5% for the detection

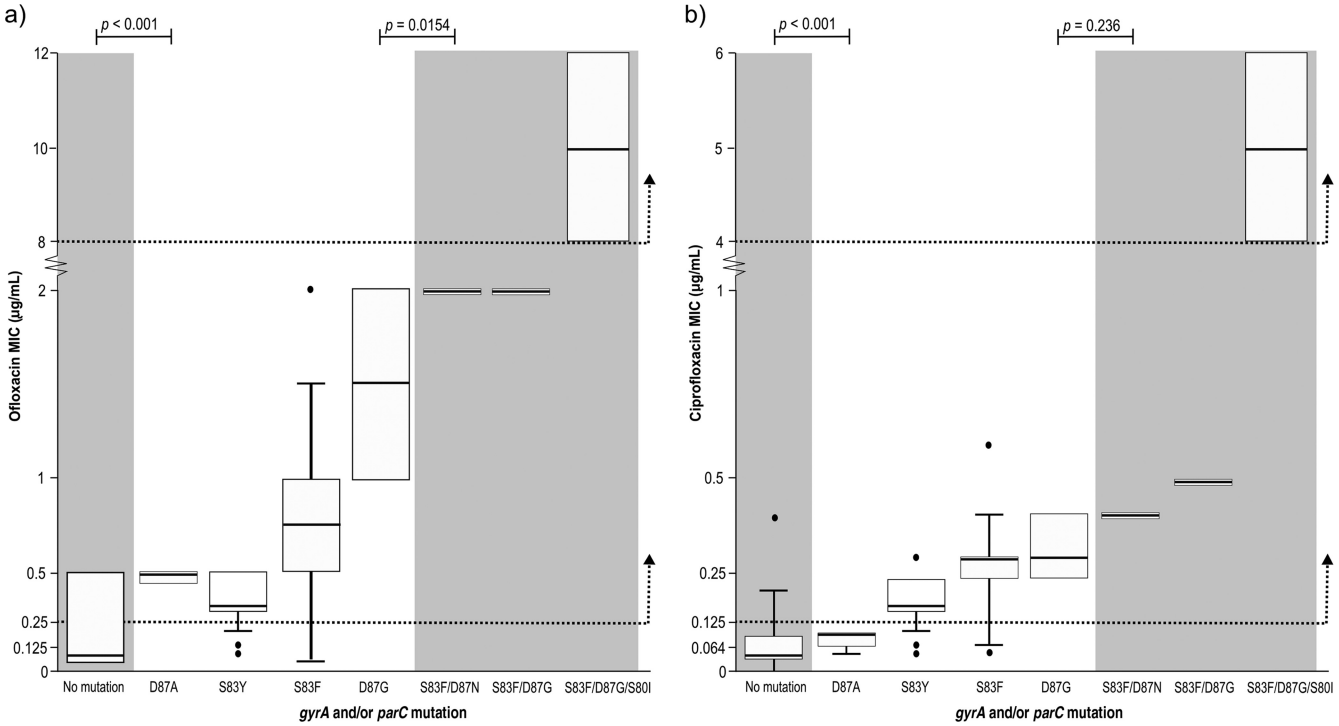

FIG. 4. The relationship of *gyrA* and *parC* mutations and the MICs to ofloxacin and ciprofloxacin in the *S*. Typhi strain isolated in Asia. Box plots (boxes relate to the 25th and 75th percentiles) relating mutations in the *gyrA* and the *parC* genes (the S80I mutation is in the *parC* gene, and the remainder are in the *gyrA* gene) to the MICs of ofloxacin (a) and ciprofloxacin (b) in 475 Asian clinical isolates of *S*. Typhi. The MICs to ofloxacin and ciprofloxacin are plotted on the *y* axis. The MICs to ofloxacin ranged from 0.016 to 12 μg/ml, and those to ciprofloxacin from 0.008 to 6 μg/ml. Median values for each mutant group are indentified by a solid line in the box. Bars demonstrate the 95% confidence interval for the groups with sufficient numbers; dots correspond to outliers. The *x* axis is subdivided into the eight different groups of *S*. Typhi strains identified and assayed, characterized as follows: no mutations in *gyrA* or *parC* ($n = 15$), D87A ($n = 2$), S83Y ($n = 46$), S83F ($n = 406$), D87G ($n = 2$), S83F/D87N ($n = 1$), S83F/D87G ($n = 1$), and S83F/D87G/S80I ($n = 2$). The upper broken lines indicate the current CLSI breakpoint recommendations for ofloxacin and ciprofloxacin. The lower broken lines correspond with the proposed breakpoints for strains with reduced susceptibility to ofloxacin and ciprofloxacin. Statistical significance was calculated between the nonmutant group and the single mutant group and between the single mutant group and the double/triple mutant group using the Student's *t* test.

of isolates with reduced susceptibility to ofloxacin and ciprofloxacin, respectively. Alternatively, using disk sensitivity testing, isolates with reduced susceptibility were detected by an ofloxacin (5-μg) disk inhibition zone diameter of ≤28 mm with a sensitivity of 94.6% and specificity of 94.2% and by a ciprofloxacin (5-μg) disk inhibition zone diameter of ≤30 mm with a sensitivity of 94.0% and specificity of 94.2%. Therefore, both methods had sufficiently high sensitivity for them to be used for screening and acceptably low levels of discrepancies (12). Disk inhibition zone size did, however, demonstrate a slightly lower specificity than nalidixic acid disk testing with this panel of isolates. Similar data for the relationship between nalidixic acid resistance and a decreased ciprofloxacin MIC have been presented for *S.* Typhi isolates in the United States (14) and India (23) and in non-*S*. Typhi *Salmonella* isolates in the United States (14) and Finland (21). For nalidixic acid-susceptible and -resistant *S*. Typhi isolates in India (23), the average disk inhibition zone sizes for ciprofloxacin were greater than those that we observed here. The non-*S*. Typhi study in Finland proposed a ciprofloxacin (5-μg) disk inhibition zone diameter of ≤37 mm as the breakpoint (21). The sensitivity of this approach was 100%, yet the specificity was only 51.9%.

In some isolates in this study, the nalidixic acid, ofloxacin, and ciprofloxacin MIC results were discrepant, in that isolates were nalidixic acid susceptible but with a reduced ofloxacin ($n = 10$) or ciprofloxacin susceptibility ($n = 22$). Similar results have been seen in other studies (13, 15, 22, 26). The clinical significance of these isolates is unclear, as there have been limited documented cases of infection with such strains treated with fluoroquinolones. It is likely that isolates that are nalidixic acid susceptible but with reduced ofloxacin and ciprofloxacin susceptibility contain resistance mechanisms other than mutations in the quinolone resistance-determining region of the *gyrA* gene. Possibilities include decreased permeability, an increase in active efflux, and the presence of plasmid-mediated genes, such as the *qnr* genes that encode a protein that protects the DNA gyrase from ciprofloxacin or *aac(6′)-Ib-cr*, an aminoglycoside-modifying enzyme with activity against ciprofloxacin (32).

The mutations that we detected in DNA gyrase genes and topoisomerase genes were consistent with previous reports (4, 6, 34, 42). The most common amino acid substitution detected was S83F, which has been found to be particularly associated with the H58 haplotype (35). This haplotype has become dominant in many areas of Asia in recent years and has also been found to have spread into Kenya in East Africa (24). Approx-

imately 20 to 25% of the isolates with a *gyrA* mutation had an MIC below the suggested breakpoints of 0.25 μg/ml for ofloxacin and 0.125 μg/ml for ciprofloxacin. The effect on the response to fluoroquinolone treatment of infection with isolates with a single *gyrA* mutation but with an MIC below the suggested breakpoints is not known. It is also possible that the isolates with a single *gyrA* mutation but an MIC above the suggested breakpoint have additional resistance mechanisms present (32).

The lack of universally observed guidelines for the detection of *S.* Typhi isolates with reduced susceptibility has meant that such isolates are frequently unrecognized by microbiology laboratories. Continued use of ciprofloxacin and ofloxacin for these infections may be driving the emergence of fully fluoroquinolone-resistant isolates of *S.* Typhi and *S.* Paratyphi A (20, 25, 34). Gatifloxacin, azithromycin, and ceftriaxone are better options for treating such infections, if the isolates also demonstrate resistance to first-line antimicrobials (7, 17, 18, 29, 31).

The use of nalidixic acid resistance as a surrogate screening test is often confusing because it is not used for the treatment of enteric fever. Furthermore, the emergence of nalidixic acid-susceptible isolates with reduced ofloxacin and ciprofloxacin susceptibility may mean that some isolates are missed. Therefore, a straightforward solution would be to modify the *S.* Typhi breakpoints to ≤30 mm and ≤28 mm for ciprofloxacin and ofloxacin, respectively. Interpretative breakpoints for the disk susceptibility tests with the antimicrobials actually used for treatment will better assist clinicians in the choice of therapy for enteric fever and will allow the collection of accurate surveillance data. Our data suggest disk breakpoints of ≤30 mm and ≤28 mm for ciprofloxacin and ofloxacin, respectively. These breakpoints have high specificity and sensitivity, permitting the detection of *S.* Typhi strains that have reduced susceptibility to ciprofloxacin and ofloxacin.

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